cores of bone spicules, apparently as a result of its earlier incorporation into matrix at higher regions of the growth plate.

The movement of 25-(OH)[^{3}H]D_3 to its target tissue reflects the demand by growing bone and cartilage for the metabolite. Quantitatively, 25-(OH)D_3 is the major metabolite found in bone (*11*) although other metabolites possess greater resorptive activity. In growing animals the demand for calcium is great because of the rapid growth of the skeleton. Although it is widely held that 25-(OH)D_3 promotes bone resorption, the histological evidence presented in this study suggests that it is involved in mineral accretion and calcium movements in epiphyseal matrix and osteoid undergoing mineralization. The long half-life of 25-(OH)D_3 combined with the availability of the metabolite to the target tissues for the 48-hour duration of the experiment did not result in its incorporation into zones of the growth plate or spicular bone where mineralization was not occurring. Growth plate chondrocytes and other bone cell populations associated with the process of mineralization of matrix were the only cells capable of incorporating the metabolite. The incorporation of 25-(OH)[^{3}H]D_3 into matrix regions suggests its ultimate association with structures or substances present at sites of initial mineralization (*12*).

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1 June 1976; revised 9 July 1976

Rod Outer Segment Disk Shedding in Rat Retina: Relationship to Cyclic Lighting

Abstract. *When albino rats are reared in cyclic light, a burst of rod outer segment disk shedding occurs in the retina soon after the onset of light. The number of large packets of outer segment disks (phagosomes) in the pigment epithelium at this time is 2.5 to 5 times greater than at any other time of day or night. The subsequent degradation of large phagosomes to smaller structures within pigment epithelial cells proceeds rapidly. The burst of disk shedding follows a circadian rhythm for at least 3 days, since it occurs in continuous darkness at the same time without the onset of light.*

Vertebrate rod photoreceptor cells continually renew their photoreceptive outer segments. The renewal process involves (i) synthesis of new membrane to form rhodopsin-containing disks at the base of the outer segment, (ii) displacement of these disks outward toward the tip of the outer segment by newly formed disks, and (iii) disposal of disks by detachment at the tip of the outer segment, followed by phagocytosis of the disks by the adjacent pigment epithelial cells to form phagosomes and degradation of the phagosomes within the pigment epithelial cell cytoplasm (*1–6*). Disks detach intermittently in groups, a process called disk shedding (*7*). Since rod outer segments maintain a relatively uniform length throughout life, the rate of disk disposal must equal the rate of disk synthesis. The rate of disposal is high, for in monkeys, rats, and mice the entire complement of rod outer segment disks is replaced every 9 to 13 days (*1, 4, 8*). It has been estimated that in the rat each pigment epithelial cell must phagocytize and degrade 25,000 to 30,000 rod outer segment disks each day (*9*). Given this high number, it is surprising to find relatively few phagosomes within pigment epithelial cells in most published studies (*3, 5, 8, 10, 11*). Either (i) degradation of ingested disks within pigment epithelial cells is rapid, (ii) phagocytized disks pass rapidly through the pigment epithelial cells and are degraded elsewhere, (iii) disks are degraded without passage into the pigment epithelial cells, (iv) most disks are phagocytized at a time when investigators usually have not taken the eyes for cytological examination (for example, at night), or (v) some combination of these factors occurs.

To explore this problem, I examined rod outer segment disk shedding in the rat retina in relation to the lighting cycle. I found that (i) a burst of disk shedding occurs soon after the onset of light in the morning and far less occurs at any other time of day or night (*12*), (ii) degradation of large phagosomes within pigment epithelial cells is rapid, and (iii) the burst of disk shedding follows a circadian rhythm since it occurs at the same time without the onset of light.

Fischer inbred albino rats were maintained in an environment with 12 hours of light and 12 hours of darkness (lights on in the morning at 0700 hours, off at 1900 hours) at a room illumination of approximately 20 to 35 footcandles (215 to 375 lu/m^2) from overhead fluorescent lamps. The illuminance levels within the cages varied from front to back, but did not exceed 15 footcandles (160 lu/m^2). The rooms were temperature-controlled at 23° ± 1°C, and the animals were fed Purina Formulab freely. At various times of the lighting cycle (*13*), the rats were killed, and their eyes were embedded in plastic, sectioned at 1.5 μm, and stained with toluidine blue (*14*). For quantification, all inclusion bodies in the pigment epithelial cell somas and the intensely staining structures among their processes (*15*) (Fig. 1a) that were greater in any dimension than 0.75 μm (half the diameter of the rod outer segments) were defined as large phagosomes and were counted as a measure of disk shedding (Fig. 2a, legend).

Some large phagosomes were present at all times of the lighting cycle. However, a sudden burst of outer segment disk shedding occurred in the morning soon after the lights were turned on (Figs. 1a and 2a). Between 0.5 and 2.25 hours after the lights came on, the number of phagosomes was 2.5 to 5 times the number found throughout the rest of the day or night (Figs. 1b and 2a). This burst of disk shedding occurred in both male and female rats ranging from 2.5 to 8 months of age.

At the peak period of disk shedding, almost every cell contained large phagosomes, and the number per 180-μm field was fairly uniform around the eye. For example, in 44 consecutive fields around a single section of an eye taken 2 hours after the onset of light, the number of phagosomes per 180-μm field was 26.3 ± 0.9 (mean ± standard error of the mean) with a range of 13 to 40. Considerably more variation occurred among animals than within an individual

eye, particularly during the peak disk shedding period and just before it (Fig. 2a).

Some of the phagosomes in the pigment epithelial cell processes were rectangular in shape, presumably representing packets of rod outer segment disks that had just detached (Fig. 1a). Many of these were about 2.5 μm in length and the largest was 4.0 μm. Since rod outer segments in the rat must renew about 2.5 μm of their length each day (*16*), some outer segments therefore shed their daily complement of disks as one large packet. Most rod outer segments must shed more than one packet of disks daily, since the mean size of phagosomes was less than 2.5 μm (*17*). Furthermore, since most phagosomes were round or ovoid in shape (Fig. 1a), a rearrangement of disk membranes (*5*) and transformation from a cylindrical to an approximately spherical shape must occur rapidly.

Most of the large phagosomes in the pigment epithelial cell somas were positioned in the apical halves of the cells (closest to the outer segments). In contrast to many large phagosomes seen apparently passing into the apical surface of the cell somas, none were seen passing through the basal surface of the cells. Therefore, large phagosomes presumably are degraded within pigment epithelial cells, at least to below criterion size, rather than passing through pigment epithelial cells for degradation elsewhere.

Degradation of large phagosomes to structures below criterion size within pigment epithelial cells proceeds rapidly. In one experiment, the number of large phagosomes peaked at 1 to 1.5 hours after the onset of light and was down to half the number by 2 hours (Fig. 2a). In another experiment, the number peaked at 1.5 to 2.25 hours and was down to about one-fourth the number by 3 hours (Fig. 2a). It therefore appears that the lifetime of large phagosomes during the peak disk shedding period is about 2 hours (*18*). Lack of data regarding the rate of degradation at later times of the day and night precludes an accurate calculation of the proportion of rod outer segment disk shedding represented by the burst in the morning.

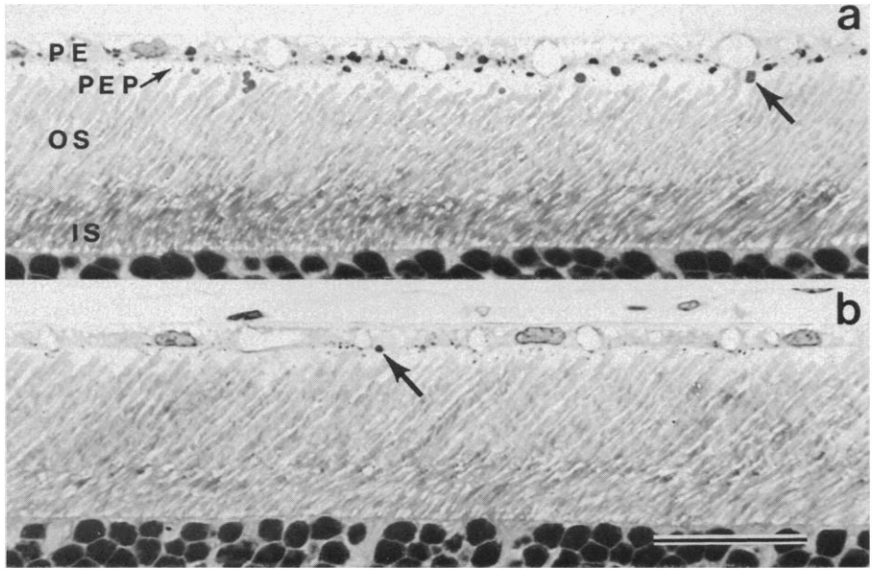

Fig. 1. Light micrographs of retinas of albino rats that were perfused at different times of the day: (a) 1.75 hours after the onset of light (0845 clock hours). Many large, intensely stained phagosomes are present in the pigment epithelial cell processes (PEP) and cell somas (PE). Most are rounded or ovoid in shape, but some are rectangular (arrow) or elongate. Abbreviations: IS, photoreceptor inner segments; OS, outer segments. (b) Nine hours after the onset of light (1600 clock hours). Only one large phagosome is present (arrow). Eyes were embedded in Epon-Araldite, stained with toluidine blue, and sectioned at 1.5 μm. Scale bar, 25 μm.

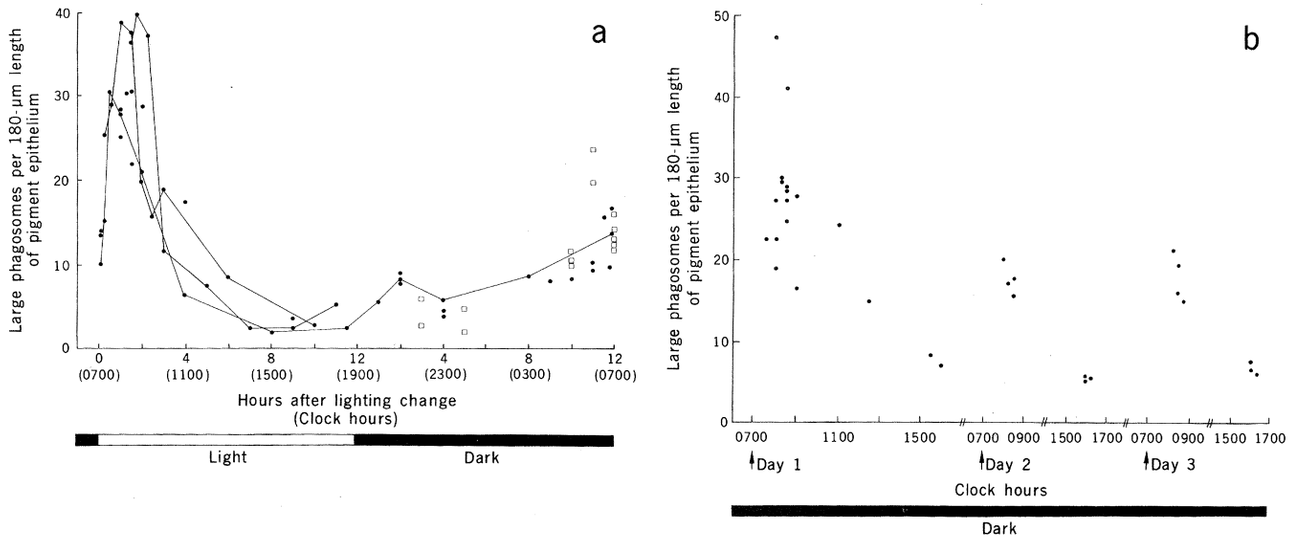

Fig. 2. Counts of large phagosomes at different times of the lighting cycle. Each point represents the mean number of phagosomes found in ten 180-μm lengths of pigment epithelium in the eye of a single animal; five consecutive 180-μm lengths were examined in the posterior retina on each side of the optic nerve head with 100× oil immersion optics. (a) Counts in cyclic lighting. The three curves represent separate experiments. The individual points represent additional animals perfused in other individual experiments. The open squares represent rats that were taken from the dark and placed in the light 1 hour before perfusion (perfusion time indicated by the squares). The dots unconnected by lines, located 1 hour before the open squares, represent control animals taken immediately from the dark in the same experiment as the animals represented by the following open squares; they were usually littermates. (b) Counts in continuous darkness. The arrows indicate when the lights would have come on if the rats had been in cyclic lighting.

Very few phagosomes below criterion size were found in the pigment cell processes, but many were present in the pigment cell somas, and they increased in number later in the day and throughout the night. These small inclusions probably were degradative stages of large phagosomes.

In order to examine the duration of dark adaptation that is required before the burst of disk shedding can occur after the onset of light, rats were removed from the dark at various times at night and were exposed to room illumination for 1 hour before perfusion. After 2, 4, or 9 hours of darkness followed by 1 hour of light, no burst of disk shedding occurred (Fig. 2a, open squares). Thereafter the results were more variable. In one experiment after 10 hours of darkness, light appeared to stimulate substantial disk shedding, but in another experiment after 11 hours of darkness, light appeared to stimulate less shedding (Fig. 2a, open squares). In these light-stimulated animals, however, the number of phagosomes present was not significantly different from that in other experimental rats still in the dark (Fig. 2a). These data indicate that for most rats, very close to a full 12 hours of dark adaptation may be required before light can induce disk shedding.

I then tried to determine whether the burst of disk shedding in the morning results from a direct effect of light on photoreceptors or pigment epithelial cells in the eye or from a systemic or humoral factor. One eye of each of seven rats was occluded in different ways (*19*), and the animals were perfused 0.5 to 1.75 hours after the onset of light. In every case the number of large phagosomes was virtually identical in both the occluded and control eyes, and the number was high, characteristic of that time of the lighting cycle. Thus, either (i) the occluding procedures did not exclude all light, (ii) the burst of disk shedding is initiated by a factor extrinsic to the occluded eye (mediated by the open eye), or (iii) the burst of disk shedding is independent of the onset of light.

In order to test the possibility that the burst of disk shedding is independent of the onset of light, no lights in the animal rooms were turned on in the morning, and no auditory cue was present that might have signaled the presumptive onset of light. Seven different experiments were carried out with one to three rats each time, and in every case the number of large phagosomes found in the 1-day extended dark period was close to the number seen when the lights ordinarily would have come on (Fig. 2b, day 1). That is, the burst of disk shedding occurred in the dark at the same time and to about the same extent as it would had the lights come on (compare Fig. 2b, day 1, with Fig. 2a).

If the burst of disk shedding follows a circadian rhythm, then it should continue in the dark (*20*). To explore this possibility, rats were perfused on the second and third days of continuous darkness at times representing the presumptive peak period of disk shedding in the morning and a presumptive low period in the afternoon. In each case the number of large phagosomes was greater in the morning than in the afternoon (Fig. 2b). Although intermediate time points have not yet been examined, the cyclic burst of disk shedding appears to continue in the dark and, for at least 3 days, to follow a circadian rhythm. Since light is not required to induce disk shedding, the phenomenon may prove to be a light-entrained circadian rhythm, but further work will be needed to establish this.

The number of phagosomes during the presumptive peak period of disk shedding was somewhat lower on the second and third days in the dark than in cyclic light (Fig. 2, a and b). This decrease may explain the fact that rhodopsin content increases 50 percent and rod outer segments elongate during the first 10 days after Fischer rats are placed in continuous darkness (*21*), since a decrease in the rate of disk disposal alone would lead to outer segment elongation.

Many circadian rhythms are controlled by serotonin-melatonin metabolism involving the pineal gland (*22, 23*). The drug reserpine abolishes some circadian rhythms by depletion of noradrenaline in the afferent nerve terminals in the pineal gland (*24*). I therefore injected rats with reserpine (5 mg/kg at 1700 hours) and perfused the animals the next morning in the expected period of extensive disk shedding 1.5 to 2 hours after the onset of light. In four rats, less than 10 large phagosomes per 180-μm field were found, and in one rat about 19 per 180-μm field were present. Thus reserpine in most animals blocks the burst of outer segment disk shedding that would have occurred soon after the onset of light. This means that the disk shedding mechanism can at least be influenced if not controlled by humoral factors. However, whether the regulating mechanism for this system is endogenous to eye structures, acts through a humoral factor, or involves the pineal gland remains to be determined and will require experiments more definitive than reserpine injection, because the site of reserpine action on this system is unknown.

The significance of the cyclic nature of rod outer segment disk shedding remains to be elucidated. The mechanism may serve to stabilize rod outer segment length in animals that undergo changes in lighting environments. To determine this it will first be necessary to learn what, if any, humoral factors control the burst of disk shedding and how this is related to the requirement for dark adaptation. Knowledge of the cyclic nature of outer segment disk shedding should now allow investigators to gain insight into the interaction of pigment epithelial cells with photoreceptor cells in the disk turnover mechanism by selecting the appropriate time of day to carry out experiments.

Rod outer segment disk shedding may be related to the onset of light in many vertebrates. In addition to that reported here for albino rats, a burst of disk shedding occurs soon after the onset of light in the frog *Rana pipiens*, in both adults (*25*) and tadpoles (*26*). Although small packets of disks may be shed during the night or day in the tadpole retina (*26*), large phagosomes are found in the adult frog pigment epithelium only after the onset of light and, in contrast to the rat, disk shedding in the frog appears to be dependent upon light stimulation and does not follow a circadian rhythm, at least for the first few days in continuous darkness (*25*).

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13. Rats examined during the dark phase of the lighting cycle were either (i) placed, at the end of the previous light phase, in their own cages into a large primate cage covered with black photographic cloth; this was in the animal room to allow access to the room without illuminating the experimental rats; or (ii) maintained in a

darkroom with double doors and environmental conditions similar to those in the animal room. They were in the light for less than 4 minutes before perfusion.

14. The rats were anesthetized with ether and killed by vascular perfusion of a mixture of 2 percent formaldehyde and 2.5 percent glutaraldehyde in 0.1*M* phosphate buffer, *p*H 7.3 to 7.4, at room temperature (22°C). The eyes of the rats were processed, embedded in an Epon-Araldite mixture, and sectioned along the vertical meridian as described elsewhere [M. M. LaVail and B.-A. Battelle, *Exp. Eye Res.* **21,** 167 (1975)].
15. Young and Bok (*2*) established that in plastic-embedded tissues stained with toluidine blue, the intensely staining inclusion bodies within pigment epithelial cells are the phagocytized packets of outer segment disks. Of those phagosomes located in the pigment cell processes in the present study, only those that stained more intensely than outer segments were counted. It was assumed that they had begun to compress their membranes, had recently detached from the rod outer segments, and were already within the pigment epithelial cells (phagosomes), but this could not be resolved by light microscopy.
16. Outer segment length in Fischer rats is about 25 μm. Total outer segment renewal time in the rat is about 10 days (*1*).
17. The long dimension of the first 100 large phagosomes encountered in the pigment epithelial cell processes and of the first 100 in the pigment cell somas was measured in sections from four rats killed during the peak disk shedding period (about 25 measured from each animal) and from four rats killed later in the day, 5 to 10 hours after the onset of light. A phagosome was defined as being in the processes if any part of it was located there. The mean size of phagosomes during the peak disk shedding period was 1.73 ± 0.05 μm (mean ± standard error of the mean) in the processes and 1.32 ± 0.05 μm in the pigment cell somas. Phagosomes present later in the day measured 1.54 ± 0.03 μm in the processes and 1.13 ± 0.03 μm in the somas.
18. The change in phagosome size may involve rearrangement of membrane rather than (or in addition to) chemical degradation and probably represents only the early phases of disk digestion. Later phases may proceed more slowly [N. F. Johnson, *Exp. Eye Res.* **20,** 97 (1975)].
19. At about 1 hour before the dark phase of the lighting cycle, either one eyelid was sutured (one rat) or one eyelid was sutured and that side of the head was painted black with a felt-tip marker (three rats). Three other rats were anesthetized with chloral hydrate about 1 hour before the onset of light, by using dim red light (BCJ ruby bulb, General Electric Co.); a piece of a standard ophthalmologic eye patch was placed over one eye, this was painted black with a felt-tip marker, and the anesthetized rats were placed on their sides with the patched side of the head buried in cage bedding until they were perfused.
20. It is generally accepted that for rhythms to be termed circadian, they must complete a cycle in about a day's length and persist in the absence of lighting cues; these are distinguished from daily rhythms that are totally dependent on environmental cues of lighting transitions (*22*).
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27. This work was supported in part by research grant EY-01202 from the Public Health Service and by research career development award EY-70871 from the National Eye Institute. I thank P. A. Ward, C. O. Gerhardt, and D. M. Colicchio for technical assistance.

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8 June 1976; revised 19 July 1976

Photoreceptor Shedding Is Initiated by Light in the Frog Retina

Abstract. *Frogs maintained on a diurnal light-dark cycle (14 hours light and 10 hours darkness) shed their rod photoreceptor outer segment tips shortly after the onset of light. Shedding is synchronous and occurs in about 25 percent of the rod photoreceptors each day. Prolonged exposure to total darkness decreases the amount of shedding, after which exposure to light results in a large burst of synchronous shedding. Thus, in the frog retina, the synchronous shedding of rod outer segment tips is shown to be directly related to light stimulation.*

Many biological events are induced by the onset of light, for example, the migration of retinal screening pigment in teleosts and amphibians (*1*) and increases in serotonin concentrations in vertebrates (*2*). Recently, LaVail (*3*) has shown that rat rod photoreceptors shed their outer segment tips according to a circadian rhythm. We have observed a similar, although not circadian, phenomenon in the frog retina, and this report describes our observations concerning the induction of photoreceptor shedding by light.

Photoreceptor rod outer segments (ROS) in vertebrate retinas are renewed through the assembly of new membrane disks at the base of the ROS. Through this process, older disks are displaced toward the apical end of the cell (*4*). Small packages of disks are shed from the tip of the ROS, thereby maintaining the outer segment at a constant length. The shed tips are engulfed by the retinal pigment epithelium (PE), forming inclusion bodies called phagosomes, which are subsequently digested and eliminated from the PE (*5, 6*). The shedding and phagocytosis of ROS tips has been described previously (*5, 6*), but until now very little was known about the initiation or regulation of these processes. We have studied the light induction of rod photoreceptor shedding in frogs previously adapted for at least 2 months to a diurnal cycle of 14 hours light and 10 hours dark.

Frogs (*Rana pipiens*, northern variety) were kept at room temperature (22° to 23°C) in a large Plexiglas chamber with a supply of constantly running water. Illumination was provided by ceiling fluorescent lamps and was at a level of 60 footcandles (645 lu/m^2) on the floor of the frog chamber. Automatic timers were used to turn room lights on at 8 a.m. and off at 10 p.m. Animals were killed at regular intervals during the diurnal cycle, and longitudinal sections through the photoreceptors and pigment epithelium were examined for the presence of phagosomes (*7*).

Just before the onset of light (7:50 a.m., Fig. 1a), no newly shed phagosomes are observed in the PE (*8*). Only small phagosomes (< 2.0 μm), presumably in the terminal stages of digestion, are seen. As is typical of dark-adapted amphibians, the granules of melanin are aggregated at the apical border of the PE cell. One hour after the onset of light (9 a.m., Fig. 1b), many newly shed phagosomes (5.0 to 6.5 μm) are seen above the tips of the ROS just inside the apical border of the PE. Most of the phagosomes are still rectangular, suggesting that they have just been shed from the ROS tips, and their dark staining appearance indicates that their degredation has already begun (*5, 9*). The outer segments of rods that have shed their tips are slightly shorter (about 10 percent) than the outer segments of those that have not, and some elongated ROS appear deeply inserted into the PE. Extensive examination of longitudinal sections at this time reveals that about 25 percent of the ROS shed their tips within the first hour after the onset of light. Ten hours into the diurnal cycle (6 p.m., Fig. 1c), the phagosomes shed after the onset of light are considerably smaller (2.0 to 4.0 μm) and are displaced toward the basal border of the PE. No new phagosomes are present at this time. At later times during the diurnal cycle (16 hours and 20 hours, not shown) only very small phagosomes with dimensions less than 2.0 μm are seen.

Figure 2 shows the time course of the appearance of new phagosomes during one complete diurnal cycle. To illustrate the light-induced synchrony of the shedding process, only rectangular phagosomes located just distal to the tips of the outer segments were counted (*8*). This temporal sequence suggests that shedding is synchronously initiated in about 25 percent of the ROS by the onset of light and is completed within the first 2 hours of the diurnal cycle, but the new phagosomes shed during the first 2 hours are, in most cases, completely digest-